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Addressing the effects of short-term exposure to TiO_2 nanoparticles in fish gills: An *ex-vivo* approach

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Abstract

With the fast increase of world-wide consumption and lack of legislation of engineered nanomaterials (ENMs), it is expected an increase of artificial production and consequently, an increase of the number of nanoparticles (NPs) that are released into the environment. Most nanoparticles present in the aquatic environment, such as those of titanium oxide nTiO₂, have effects on histopathological alterations on fish, creating several implications on their health, and, consequently influencing aquatic environment status.

The effects caused by the exposure to two realistic concentrations of nTiO₂ (20 and 200 µg L⁻¹, plus controls) were evaluated in gills of fish (*S. senegalensis*), through a short-term *ex vivo* approach, meaning that exposure was accomplished after dissection of gills from the animals. Alterations in gills were analysed, such as the formation of metal deposits and the specific alterations to gas-exchange epithelial and chloride cells. Standard histological techniques were coupled with fluorescent techniques to assess aforesaid alterations. Quantitative and semi-quantitative approaches were employed.

Overall, the main alterations observed in gill exposed to nTiO₂ treatments were epithelial lifting, chloride cells autolysis and goblet cell hypertrophy. Higher severity and dissemination of alterations was observed for gills exposed to the highest concentration (200 µg nTiO₂ L⁻¹). In accordance, the gill global histopathological condition indice (I_h) increased with the increase of nTiO₂ concentration in water at T₂ and T₄. The number of CC (Chloride cells) and GC (Goblet cells) per interlamellar space also increased with the exposure to nTiO₂, at T₂, however without a clear relationship with the concentration. Metal deposits were found in gill macrophages, distributed consistently through all treatments, failing to demonstrate any cause-effect relation between concentrations and time of exposure.

Overall, the present study indicates that under ecologically-relevant concentrations of nTiO₂ caused moderately histopathological lesions in gills of *S. senegalensis*. Although, the alterations in mucocytes indicated responses to the challenge, the exposure to TiO₂ promoted osmotic imbalance. The present *ex vivo* study significantly contributed to define further procedures to nanotoxicity studies.

Resumo

Com o rápido aumento do consumo a nível mundial e a falta de legislação de nanomaterias produzidos (ENM), espera-se um aumento da produção artificial e, conseqüentemente, um aumento da quantidade de nanopartículas (NPs) libertadas para o meio ambiente. A maioria das nanopartículas presentes no ambiente aquático, como as de óxido de titânio $n\text{TiO}_2$, originam alterações histopatológicas nos peixes, criando várias implicações na saúde e, conseqüentemente, têm efeito sobre a saúde do meio aquático.

Os efeitos causados pela exposição a duas concentrações realistas e ambientalmente relevantes de $n\text{TiO}_2$ (20 e 200 $\mu\text{g L}^{-1}$, mais controlos) foram avaliados em brânquias de peixes (*S. senegalensis*), através de uma abordagem ex vivo de curto prazo, o que significa que a exposição foi realizada após a dissecação de brânquias dos peixes. Foram analisadas alterações em brânquias, como a formação de depósitos metálicos e alterações de trocas gasosas em células epiteliais e de cloro. Técnicas histológicas padrão foram acopladas com técnicas fluorescentes para avaliar as alterações acima mencionadas. Foram utilizadas abordagens quantitativa e qualitativas.

Em geral, as principais alterações observadas nas brânquias expostas aos tratamentos foram o descolamento epitelial, autólise das células de cloro e hipertrofia das células de muco, onde 200 $\mu\text{g nTiO}_2 \text{ L}^{-1}$ induziu alta severidade e disseminação. O índice de condição histopatológico global (Ih) aumentou a exposição às $n\text{TiO}_2$ na água em T₂ e T₄. O número de CC (células de cloro) e GC (células de muco) por espaço interlamelar também aumentou após as brânquias terem sido expostas a $n\text{TiO}_2$, porém sem uma relação clara com a concentração. Os depósitos de metal foram encontrados em macrófagos, distribuídos de forma consistente através de todos os tratamentos, não demonstrando qualquer relação causa-efeito entre as concentrações e o tempo de exposição.

Em geral, o presente estudo indica, que, sob concentrações ecológicamente relevantes de $n\text{TiO}_2$, existem lesões histopatológicas moderadas em brânquias de *S. senegalensis*. Embora as alterações nos mucócitos indicassem resposta ao desafio, a exposição de $n\text{TiO}_2$ promoveu um desequilíbrio osmótico. O presente estudo contribuiu significativamente para definir procedimentos adicionais em estudos ex vivo de nanotoxicidade.

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Abbreviation List

CC	Chloride cells
CCA	Chloride cell autolysis
ENM	Engineered nanomaterials
FL	Gill filament
GC	Goblet cells
HGC	Hypertrophied goblet cells
IS	Interlamellar space
I_h	Histopathological indice
I₁	Histopathological indice for the circulatory disturbances/Inflammatory response response pattern
I₂	Histopathological indice for the regressive response pattern
I₃	Histopathological indice for the progressive response pattern
LM	Lamellae
MMC	Melanomacrophage
MD	Metal deposits
NFR	Nuclear Fast Red
NM	Nanometer
NP	Nanoparticle
nTiO₂	Titanium dioxide nanoparticles
ROS	Radical Oxygen Species
PC	Pillar cells
PCB	Polychlorinated biphenyl
TEM	Transmission Electron Microscopy
UV	Ultraviolet

1. Introduction

Nanoparticle (NPs), defined as particles with a size ranging between 1 and 100 nm on at least one dimension, have become rapidly introduced on the global economy, regarding nanotechnology products with a worth estimated at \$26 billion, and expected to reach about \$65 billion by 2019 (Winkler, 2016), overtaking the market at an increasing pace. Due to their nanoscale size, they have greater surface to volume ratio than bulk forms, which offers them unique physicochemical properties, namely larger reactivity and mobility (Rauscher et al., 2014), leading to numerous applications from biomedical to electronic science, cosmetic and pharmaceutical industries and environmental remediation.

Engineered nanomaterials (ENMs) are synthesized worldwide in various forms, shapes and sizes allowing adjustment to different functionalities but also increasing the scale by which entrance in the ecosystem occur. With the increase of world-wide consumption and lack of legislation of NPs, it is expected an increase of artificial production and consequently, an increase of the number of NPs that are released into the environment (Piccinno et al., 2012; Canesi et al., 2009). Their presence in the aquatic environment is likely to be non-uniform with higher concentrations in near-shore waters which are more impacted by run-off, wastewater discharge, and proximity to human populations (Gottschalk et al., 2011). When entering the aquatic environment, NPs will be subjected to several transformations, like dissolution, aggregation and sedimentation that will change their physico-chemical properties, which may influence their bioavailability and toxicity to aquatic organisms (Piccinno et al., 2012). Consequently, concerns about the safe use and environmental impacts of NPs in aquatic systems have been increasing and are essential for Environmental Risk Assessment in order to ensure the correct management of associated risk and, the safety of these manufactured materials, which are still poorly understood (Piperigkou et al., 2016; Toropova and Toropov, 2013).

Among all nanoparticles, Titanium dioxide nanoparticles (nTiO_2) are widely used due to their unique properties like, chemical composition, surface structure, solubility, shape and aggregation and photocatalytic properties, mainly ultra-violet (UV). These NPs are widely used in the production of industrial sunscreens, toothpaste, shampoo, paper products, plastic, ink, food and food colour, as additive in paint and building materials, in surface coatings and water treatments to destroy chemicals such as PCB, pesticides and other complex organic contaminants (Nel et al., 2006; Chen and Mao, 2007, Mu and Sprando, 2010). Although knowledge about the real concentrations of nTiO_2 is lacking, they have been detected in soil, surface water, wastewater and sewage sludge (Li et al., 2015) and the usual estimated concentrations ranged from 0.7 to 24.5 μgL^{-1} (Mueller and Nowack, 2008; Pérez et al., 2009). It is predicted that the concentration in surface water is 0.02 μgL^{-1} , while values up to 4 μgL^{-1} are found in sewage treatment waters (Gottschalk et al., 2009).

The toxicity of nTiO₂ to aquatic organisms is commonly attributed to three mechanisms: i) physicochemical stress in organs and tissues (cytotoxicity) caused by their size, shape and surface properties (Libralato et al., 2013; Vale et al., 2014); ii) chemical toxicity associated with NPs capacity to adsorb contaminants in the media (Pettibone et al., 2008; Cho et al., 2010); iii) phototoxicity associated with the formation of reactive oxygen species when nTiO₂ are irradiated by UV light (Garvas et al., 2015). Some studies revealed several effects of nTiO₂ in freshwater species such as: decreased immune response against pathogens (Blaise et al., 2008), increased bioaccumulation of contaminants associated with NPs and possible combined effects with other pollutants (Sun et al., 2009; Hu et al., 2011; Fan et al., 2012; Tan et al., 2012), immunotoxicity, cytotoxicity and oxidative stress as well as physiological and reproductive alterations (Menard et al., 2011; Jovanović and Palić, 2012; Boyle et al., 2013; Diniz et al., 2013; Vale et al., 2014). It was also reported that prolonged exposure of fish to nTiO₂ induced biochemical and histopathological alterations in their gills, liver and intestines (Blaise et al., 2008; Boyle et al., 2013; Federici et al., 2007). Similar effects have been observed in invertebrate marine species (Blaise et al., 2008; Boyle et al., 2013; Federici et al., 2007).

Despite extensive research on freshwater species, few studies have been focusing on marine organisms. Despite their diversity and abundance, most studies have examined effects on a few representative species including *Pseudomonas* spp. (bacteria), *Thalassiosira* spp. (diatoms) and *Mytilus* spp. (mussels). For example, an *in vivo* study exposing *M. galloprovincialis* to nTiO₂ revealed increased oxidative stress in digestive gland and, also effects on gene transcription (Barmo et al., 2013). Studies with marine fish are also scarce. Injection of *Trachinotus carolinus* with nTiO₂ led to genotoxicity and accumulation of nTiO₂ in the kidney, gills, liver and muscle (Vignardi et al., 2015). Another study, reported sub-lethal adverse effects of nTiO₂ on the early developmental stages of the brackish water species *Oryzias latipes* (Paterson et al., 2011). In fact, despite their relevance, estuarine and brackish water species, are seldom used in NP experiments. Indeed, these species may be more subjected to NPs toxicity, since the dynamic estuarine environment may increase the speciation of dissolved ions and the complexation of insoluble NPs, leading to sedimentation, and potential re-suspension after remobilization of sediments (Baker et al., 2014).

Among other marine organisms, fish is often chosen as model organism, due to their ecological and economical relevance. The gills are considered a target organ to assess the toxicity of several contaminants, because they are the main entry route of waterborne toxic substances and comprise important physiological functions, such as, gas exchange and ion transport (Stentiford et al., 2003; Riba et al., 2004, 2005; Costa et al. 2009, 2010, 2011). Thus, fish gill histopathology is regarded as an important tool in this research area being used in a growing number of studies, both *in situ* and *ex situ* (Stentiford et al., 2003; Riba et al., 2004, 2005; Costa

et al. 2009, 2010, 2011). However, mechanistic data on the etiology of gill histopathological lesions and alterations in fish exposed to ecologically-relevant concentrations of NPs is scarce.

In Environmental Toxicology, histopathological analyses has been tested and proposed as an efficient and sensitive biomarker to assess the health and environmental status of organisms exposed to environmental chemicals (Teh et al., 1997; Handy et al., 2002; Wester et al., 2002; Stentiford et al., 2003), mostly because they reflect organism health more realistically than biochemical biomarkers and can thus be better extrapolated to community- and ecosystem-level effects of toxicity (Au, 2004). Nevertheless, the establishment of cause-effect relationships between pathology and contamination is difficult through qualitative approaches. In order to fill this constrain, histopathological indices were developed providing numerical data based on a semi-quantitative approach, linking the qualitative and quantitative approaches. These indices are built taking the biological significance and, also the dissemination of histopathological changes, thus conferring also a wider biological significance (Costa et al., 2009; Vethaak and Wester, 1996). Still, fish histopathology is far from being standardized, having problems in establishing cause-effect relationships in higher vertebrates, as well as the lack of specificity of most biomarker candidates. Furthermore, there are yet few studies with fish exposed to environmentally realistic concentrations of TiO_2 and even fewer concerning histopathology.

In vitro studies are considered the fastest and most convenient approach to assess the toxicity of NPs (Park et al., 2009), due to minor ethical issues, easier logistics and decreased confounding effects compared to *in vivo* studies. These methodologies are well-established being, therefore, an important tool for mechanism-oriented studies. However, one constrains of this approach is usually attributed to the absence of intercellular interactions. Thus, as suggested by Valant & Drobne (2011) in a study using isolated digestive glands (hepatopancreas) of a model invertebrate to assess the biological reactivity of nTiO_2 , *ex vivo* test systems arise as a suited approach to the fast screening of the biological potential of nanoparticles. Conversely, this model is poorly explored in the environmental toxicology, even knowing its potential as a tool to asses NPs toxicity.

2. Objectives

The present work aims to assess the histopathological effects caused by the exposure of flatfish (*S. senegalensis*) to realistic concentrations of nTiO₂, through a short-term *ex vivo* approach, meaning that exposures were accomplished after dissection of gills from the fish.

Specifically, this Thesis intends to:

1. Identify histopathological lesions and alterations in the gills of *S. senegalensis* after the exposure to n TiO₂;
2. Assess time- and dose-responsiveness of effects from exposure;
3. Establish a link between potential and detectable n TiO₂ in gills and the effects observed;
4. Evaluate if *ex vivo* assays with fish gills are an appropriate method to assess the effects of NPs.

S. senegalensis is a benthic teleost of important value for fisheries and aquaculture in Southern Europe. The species inhabits soft bottoms of coastal areas, especially estuaries, which function as breeding and nursing grounds, where it feeds on small invertebrates (Cabral and Costa, 1999; Cabral, 2000). Combined with its relative abundance, these characteristics contribute to the species projected value as a sentinel for environmental contamination (Jiménez-Tenorio et al., 2007), being successfully employed in field surveys (Stentiford et al., 2003) or laboratorial exposures to sediments (Riba et al., 2004, 2005; Jiménez-Tenorio et al., 2007; Costa et al., 2008), surveying histopathology and other effects and responses to toxicity (Costa et al. 2009, 2010, 2011).

3. Materials and methods

3.1. Test solutions

The nTiO₂ nanoparticles (99.5%) were commercially obtained from aerioxide® P25. These NPs are pure titanium dioxide with high specific surface area and a mixture of anatase and rutile crystal structure. A stock solution with the concentration of 4 mgL⁻¹ was prepared with seawater and then diluted with seawater to two target concentrations (20 and 200 µg nTiO₂ L⁻¹).

3.2. Bioassays

Two independent experiments were performed at University of Aveiro laboratory (Fig. 3.1). The bioassays consisted of ex vivo experiments using excised gills from *S. senegalensis* which were exposed to the test solutions for 2h (T₂) and 4h (T₄), experiment A and B respectively. The fish were obtained from Aquacria aquaculture and acclimatized for 15 days.

For each experiment: 24 tubes were filled with 4ml of each test solution (sea water, 20 and 200 µg nTiO₂ L⁻¹) and placed under constant gentle shaking; eight fish (±140 g) were collected from the rearing system and the gills were immediately excised; the eight arches obtained from each fish were distributed among the three treatments, to obtain two arches per tube (figure 1). The two remaining arches were immediately fixed in Bouin-Hollande (T₀ gill samples). After the exposure times (T₂ and T₄) the gill samples were also fixed. Afterwards, samples were washed in water and archived in 70% v/v ethanol, for subsequent histopathological analysis.

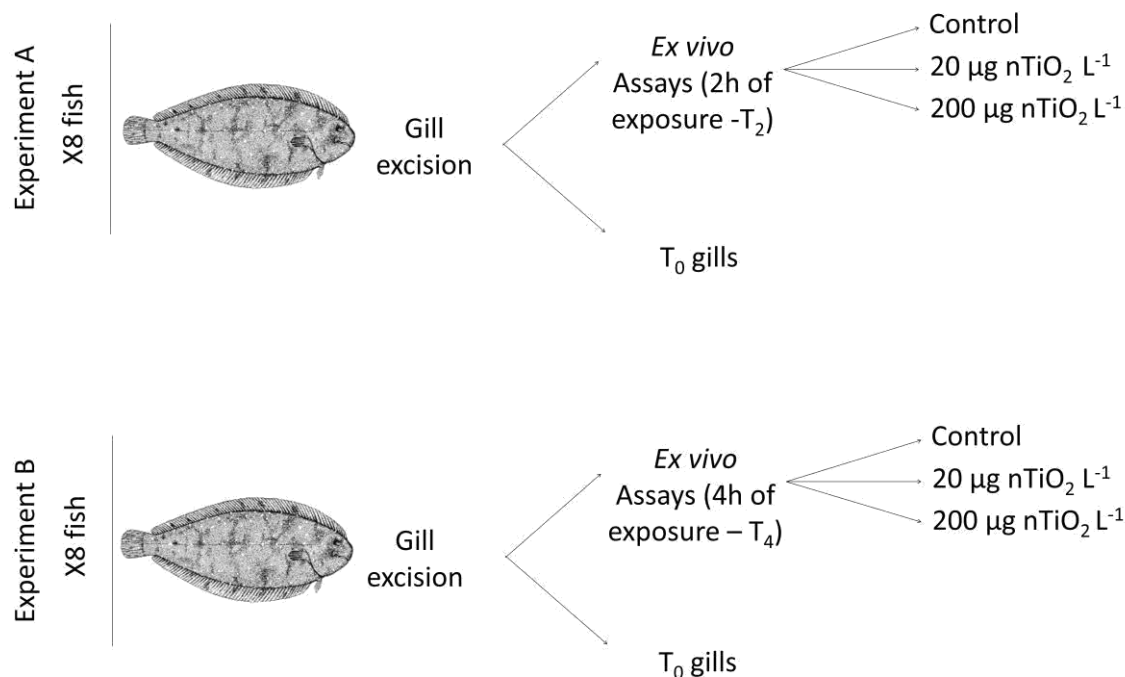


Figure 3.1 – Scheme of the two-independent ex vivo bioassays, experiment A and B. Gill of *S. senegalensis* were exposed for 2 and 4h to two concentrations of nTiO₂. For each experiment, gills were excised from fish (eight in total) and the branchial arches (eight) were randomly distributed among the three treatments (control, 20 and 200 µg nTiO₂ L⁻¹) to obtain two arches per treatment. The remaining two arches were fixed immediately after the excision and correspond to T₀ gill samples.

3.3. Histology

Gill samples were prepared for histological analyses. In brief: the samples were dehydrated with a progressive series of ethanol (95% and 100% v/v respectively), intermediate impregnation with xylene and embedded in paraffin. Gills sections of 5µm thick, were obtained using a rotary microtome (Leica JUNG RM2035) and, at least 8 sections per slide were obtained. The slides were dewaxed and rehydrated with progressive series (30 seconds each) of Xylene, 100%, 95%, 70% v/v Ethanol and distilled water for 6 minutes and 3 distinct staining protocols were applied: i) Nuclear Fast Red, during 10 min, as contrastant, to detect nanomaterial deposits (Costa and Costa, 2012); ii) Acridine Orange Fluorochrome (for 40 min), to identify mitochondria in chloride cells (Costa and Costa, 2012), and iii) Standard Tetrachromic procedure, combining Alcian Blue (30 min), Weigert's Haematoxylin (10 min) and Van Gieson's dye (6 min), following Costa & Costa (2012) and Martins et al. (2016), for the detections of evident lesions and alterations and to reveal mucous substances and chloride cells respectively.

After each staining, the slides were cleared with Xylene and mounted with DPX resinous media (BHD, Pool, UK) and then rested for 24 hours. Four slides with (6-12 sections each) were prepares for each sample. A Leica DMLB microscope, equipped with a DFC480 digital camera, was used for the microscopic analysis. Image processing and analysis was performed with the software IrfanView, Image J (Schneider et al., 2012).

3.4. Gill Histopathology

Quantitative analyses were performed in gill samples by counting the chloride cells (CC), goblet cells (mucocytes) (GC) and metal deposits (MD) per interlamellar space (IS). Data were expressed as mean number of each gill cell metrics per interlamellar space.

Semi-quantitative histopathological conditions indices were estimated for each individual, according to their biological significance of each surveyed alteration within the surveyed organ (score). The weight of alterations ranged between 1 (low severity) and 3 (high severity) and the score ranged from 0 (absent alteration) to 6 (diffuse alteration). The respective pathological alterations were classified into three reaction patterns: circulatory disturbance/inflammatory responses, regressive and progressive alterations. In table 3.1 are presented the histopathological alterations surveyed and their respective weights. The histopathological indices were estimated trough the following formula proposed by (Costa et al., 2013):

$$I_h = \frac{\sum_1^j w_j a_{jh}}{\sum_1^j M_j}$$

where w_j is the weight of the j th histopathological alteration; a_{jh} is the score attributed to the h th individual for the j th alteration and M_j is the maximum possible score, which normalizes I_h to a value between 0 and 1. As well as the global histopathological indice (I_h), partial indices including the different reaction patterns, I_1 (circulatory disturbances/inflammatory responses), I_2 (regressive changes) and I_3 (progressive alterations) were also calculated.

In order to assess the accuracy of the observation, a blind review was performed at the end of the histopathological analyses in 25% of the samples with a 12.8% error.

Table 3.1 – Histopathological alterations observed in the gills of *S.senegalensis* and their respective condition weights (w)

Reaction pattern	Histological alterations	w
<input type="checkbox"/> Circulatory disturbances/Inflammatory responses <input type="checkbox"/> Regressive	<input type="checkbox"/> Infiltration of inflammatory cells	1 ^b
	<input type="checkbox"/> Epithelial lifting	1 ^b
	<input type="checkbox"/> Structure alterations of lamellae	1 ^b
	<input type="checkbox"/> Goblet (mucous) cells	2 ^b
	degeneration	2 ^c
	<input type="checkbox"/> Chloride cells autolysis	2 ^b
	<input type="checkbox"/> Apoptosis	
<input type="checkbox"/> Progressive	<input type="checkbox"/> Epithelial cell hypertrophy	2 ^b
		2 ^b
	• Goblet cell hypertrophy	
		2 ^a
	• Interlamellar/ epithelial hyperplasia	

^a Bernet et al. (1999)

^b Costa et al. (2009)

^c Martins and Costa (2015)

3.5. Statistical analyses

Shapiro-Wilks tests were performed to analyse the normality of the data obtained, followed by Levene test to analyse the homogeneity of variances. Due to the invalidation of at least one of the tests, the non-parametric Kruskal-Wallis one-way ANOVA followed by the Mann-Whitney *U* post hoc test was employed for temporal comparisons as well as for gender and age comparisons. Spearman's rank statistic was applied to establish correlations. The statistical analysis was assessed using Statistica 8.0 software (Starsoft, USA).

4. Results

4.1. Gill histopathology

Excised gills of *S. Senegalensis* (T_0 gill samples) of each experiment (2h and 4 h of exposure) exhibited normal morphology (Fig. 4.1A), presenting well-defined lamellae attached to filaments, in accordance with previous descriptions for gills of juveniles of the species (Costa et al., 2009; Costa et al., 2010). The gill epithelium included pavement cells, goblet cells (mucocytes) and chloride cells, the latter type cells mostly located in the interlamellar space. The controls revealed some alterations of the epithelia relatively to T_0 gills, namely, epithelial lifting and alterations of the epithelia structure (Fig. 4.1B).

These alterations were also observed in gill exposed to $nTiO_2$ treatments, however with higher severity and dissemination (Fig. 4.1C). Gills exposed to $200 \mu g nTiO_2 L^{-1}$ for 2 h and 4 h presented, in general, higher severity and diffusion of lesions. Among the most significant alterations, chloride autolysis and goblet cell hypertrophy were the most remarkable. Chloride cells (cc) were mainly found in the interlamellar space (as expected), however the cells evidence autolytic processes: vacuolated appearance of chloride cells indicating fluid retention and possible swelling and, also a nucleus compressed against the plasmalemma (Fig. 4.1C, inset). This alteration was mostly observed in gill exposed to both concentrations for 2h. Goblet cell hypertrophy (Fig. 4.1D, inset) was also observed in all $nTiO_2$ treatment, although gill exposed to $200 \mu g nTiO_2 L^{-1}$, at T_2 presented higher severity and dissemination (Fig. 4.1D). It was also observed moderate apoptosis (Fig. 4.1C, inset), presence of inflammatory cells (Fig. 4.1F) and interlamellar hyperplasia (Fig. 4.1D) in $nTiO_2$ treatments, although with low severity and dissemination. Metal deposits were found in gill macrophages (forming black deposits), distributed consistently through all treatments (Fig. 4.1F), failing to demonstrate any cause effect with higher concentrations and time of exposure.

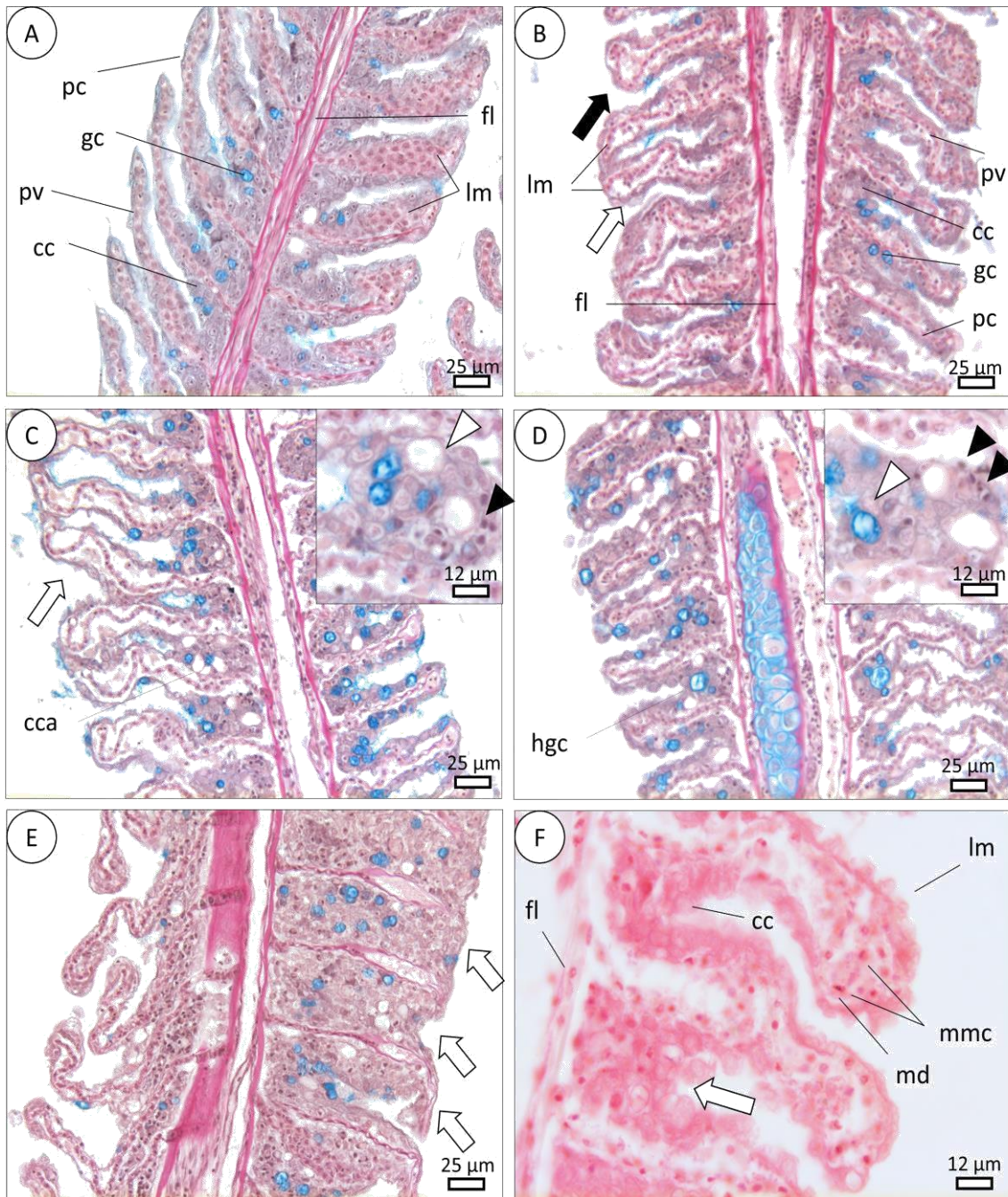


Figure 4.1 – Gill histopathological sections of *S. senegalensis* stained with tetrachrome stain (A,B,C,D,E) and NFR (F); (A) Overall aspect of the morphology of the gills obtained from T_0 gill samples (gill excised and immediately fixed), exhibiting normal gill filament (fl), lamella (lm), pillar cells (pc), pavement cells (pv), chloride cells (cc) and goblet cells (gc); (B) Overall aspect of control samples, exhibiting epithelial lifting (white arrowhead) and structure alterations (black arrowhead); (C) Gill exposed to $200 \mu\text{g nTiO}_2 \text{ L}^{-1}$ at T_4 showing epithelial lifting (arrowhead) and chloride cell autolysis (cca); inset chloride cell autolysis (white arrowhead) and apoptosis (black arrowhead); (D and inset) hypertrophied goblet cells (arrows) and apoptosis (black arrowhead) present in gills exposed to $200 \mu\text{g nTiO}_2 \text{ L}^{-1}$; (E) interlamellar hyperplasia (arrows) observed in gills exposed to $20 \mu\text{g nTiO}_2 \text{ L}^{-1}$ treatment at T_2 ; (F) metal deposits (white arrow), forming black deposits, macrophages (mmc) and interlamellar hyperplasia (black arrow) observed in gills exposed to $20 \mu\text{g nTiO}_2 \text{ L}^{-1}$ treatment at T_4 .

4.2. Quantitative histopathological measurements

Significantly higher number of chloride cells per IS were obtained in gills exposed to nTiO₂ treatments comparing to T₀ gill samples and control treatment (Mann-Whitney *U*, $p < 0.01$), at T₂ (Fig. 4.2A). Conversely, no significant differences were observed between T₀ gills and controls and between all treatments.

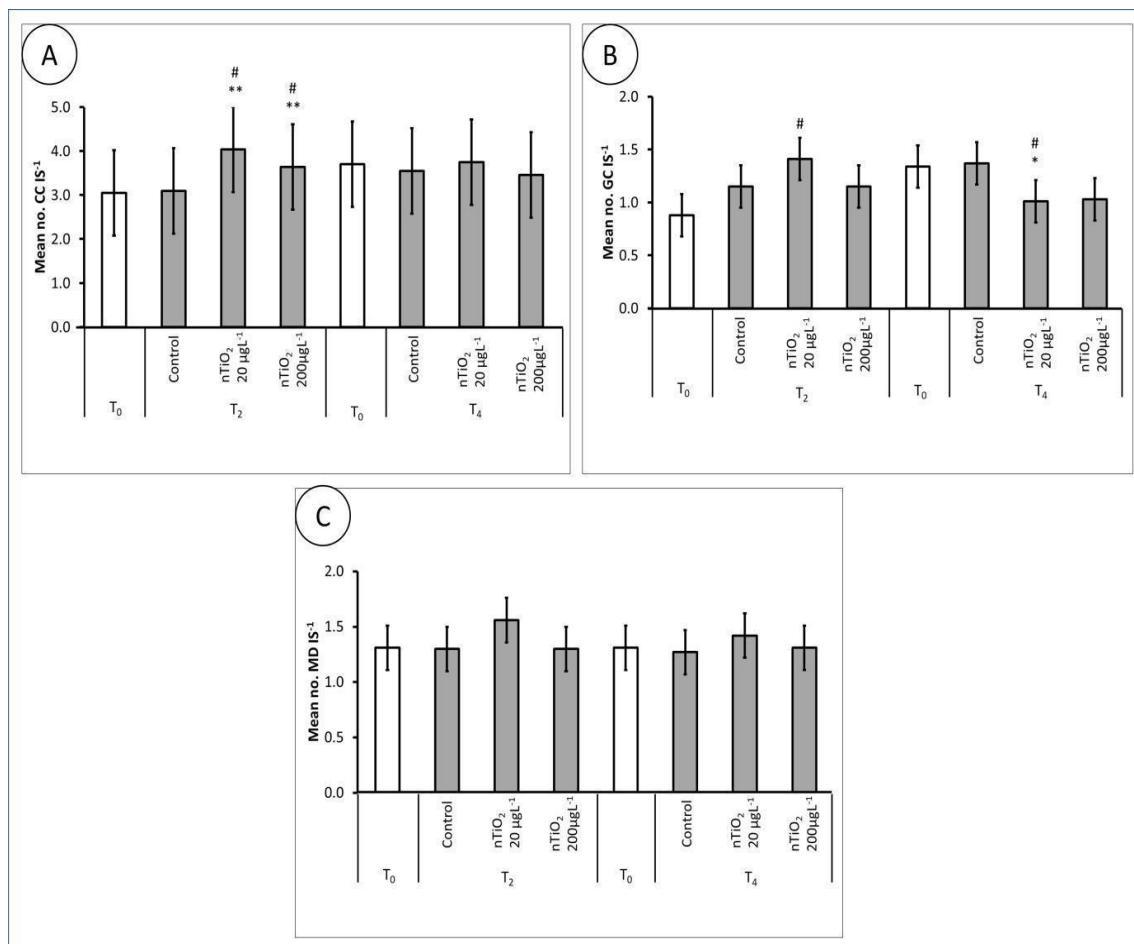


Figure 4.2 - Comparison of the mean number of chloride cell (A), goblet cells (B) and metal deposits (C) per interlamellar space (IS) between gills exposed to control, 20 and 200 µg nTiO₂ L⁻¹ treatments, for 2 (T₂) and 4 h (T₄) and T₀ gill samples (gill excised and immediately fixed). * and ** indicate significant differences between treatments and its respective control, $p < 0.05$ and $p < 0.01$, respectively (Mann-Whitney *U* test). # and ## mean significant differences to its respective T₀, $p < 0.05$ and $p < 0.01$ respectively (Mann-Whitney *U* test). Error bars indicate standard deviation.

The number of goblet cells per IS were significantly higher in 20 µg nTiO₂ treatment T₂ (Fig. 4.2B). On the other hand, at T₄, the number of GC/IS decreased in gills exposed to nTiO₂ relatively to T₀ samples and control treatment, being significantly different in gill exposed to 20 µg nTiO₂ L⁻¹ (Mann-Whitney *U*, $p < 0.05$).

No significant differences were observed between T_0 gill samples and treatments (control, 20 and 200 $\mu\text{g nTiO}_2 \text{ L}^{-1}$) for both times of exposure (Fig 4.2C).

4.3. Gill Histopathological condition indices

Gills exposed to nTiO_2 for 2h revealed significantly higher global histopathological condition indice (I_h) compared to T_0 and control gills (Fig.4.3). At this time point (T_2) and considering exposures to nTiO_2 , the concentration of 200 $\mu\text{g nTiO}_2 \text{ L}^{-1}$ yielded the highest I_h value, being significantly different from the respective T_0 gill samples and from the controls (Mann-Whitney U , $p < 0.01$). At T_4 , the highest I_h value was also registered for gills exposed to 200 $\mu\text{g L}^{-1}$ nTiO_2 , being significantly different to T_0 gills and 20 $\mu\text{g nTiO}_2 \text{ L}^{-1}$ treatment (Mann-Whitney U , $p < 0.05$).

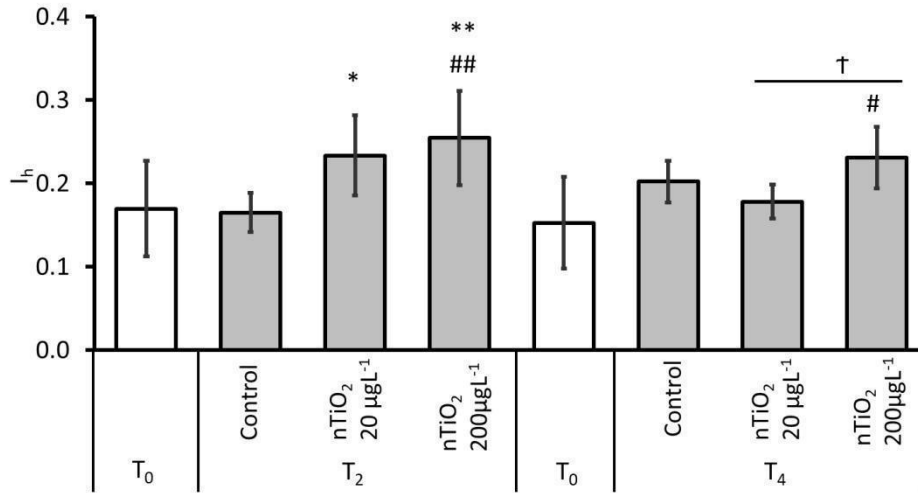


Figure 4.3 - Comparison of the average histopathological indice (I_h) between gills exposed to control (C) and 20 and 200 $\mu\text{g nTiO}_2 \text{ L}^{-1}$ treatments for 2 (T_2) and 4 h (T_4) and basal I_h from gills at the beginning of the experiment (T_0 gills excised from fish and immediately fixed). * and ** indicate significant differences between treatments and its respective control, $p < 0.05$ and $p < 0.01$, respectively (Mann Whitney U test). # and ## mean significant differences to its respective T_0 , $p < 0.05$ and $p < 0.01$ respectively (Mann Whitney U test). † indicate significant differences between nTiO_2 treatments. Error bars indicate standard deviation.

In general, the average histopathological indices score obtained for the different reaction patterns exhibited similar variations as the global indices. Accordingly, gills exposed to nTiO_2 treatments for 2h (T_2) yielded higher of each individual indices (I_1 , I_2 , and I_3) comparing to T_0 and control gills (Fig. 4.4), however, the significant differences were only obtained for I_2 and I_3 indices (Fig. 4.4B, 4.4C), Mann-Whitney U , $p < 0.05$ and $p < 0.01$, respectively. No significant differences were observed between nTiO_2 treatments, however, the results show a tendency: the increase of indices for each reaction pattern with the increase of nTiO_2 concentrations in water. At T_4 , the pattern was similar to T_2 , for I_2 indice. Conversely, the progressive alterations reaction pattern indice (I_3) were significantly lower for 20 $\mu\text{g nTiO}_2 \text{ L}^{-1}$, relatively to T_0 samples at both sampling times (Fig. 4.4C). Significant differences between nTiO_2 treatments were only observed for I_3 , at T_4 .

significantly higher values for nTiO₂ treatments relatively to T₀ samples ($p < 0.05$). Conversely, the progressive alterations reaction pattern partial indice (I_3) were significantly lower for 20 μg nTiO₂ L⁻¹, relatively to T₀ samples (Fig. 4.4C). Significant differences between nTiO₂ treatments were only observed for I_3 , at T₄. No significant differences were observed regarding circulatory disturbances/inflammatory (I_1), for both sampling times (Fig. 4.4A).

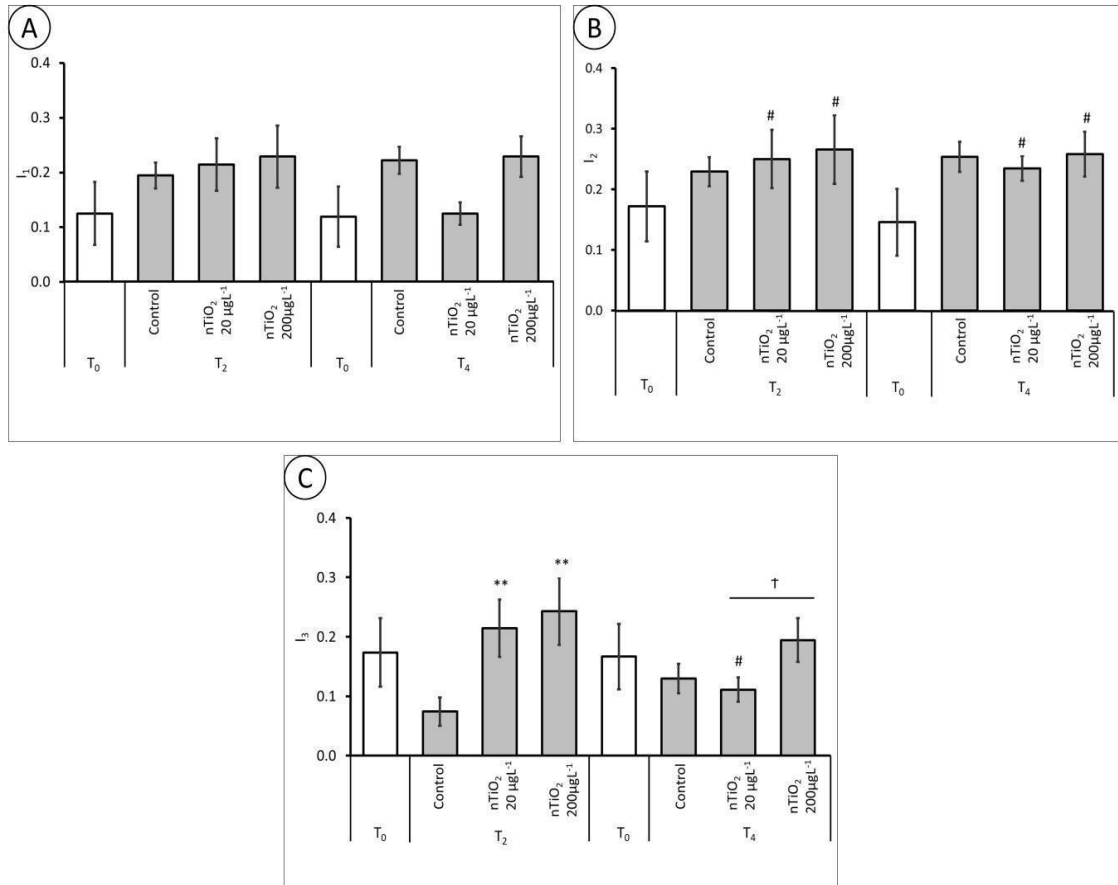


Figure 4.4 – Comparison of gill histopathological indices for each reaction pattern between gills exposed to control (C) and 20 and 200 μg nTiO₂ L⁻¹ treatments for 2 (T₂) and 4 h (T₄) and gills at the beginning of the experiment (T₀ gills excised from fish and immediately fixed). Circulatory disturbances/Inflammatory response (I_1); Regressive alterations (I_2); Progressive alterations (I_3). * and ** indicate significant differences between treatments and its respective control, $p < 0.05$ and $p < 0.01$, respectively (Mann Whitney *U* test). # and ## mean significant differences to its respective T₀, $p < 0.05$ and $p < 0.01$ respectively (Mann Whitney *U* test). † indicate significant differences between nTiO₂ treatments. Error bars indicate standard deviation.

4.4. Correlation analyses

H Spearman's statistic showed the highest correlations between I_1 , I_3 ($R=0.71$, $p<0.05$) and I_2 ($R=0.79$, $p<0.05$), demonstrating that progressive and regressive alterations were fundamental to increase the histopathological indice. Regressive responses (I_2) showed the highest correlations were obtained with apoptosis ($R=0.67$, $p<0.05$) and epithelial lifting ($R=0.66$, $p<0.05$). For the progressive responses (I_3), goblet cell hypertrophy ($R=0.79$, $p<0.05$) was the main alteration to affect this specific histopathological indice. Neither goblet cell, chloride cell

nor metal deposits mean value were highly correlated with any of the most significant alterations found through the *Spearman's* statistic.

5. Discussion

The present work is the first *ex vivo* experiment which used gills of *S. senegalensis* to assess the toxicity of nTiO₂. Few studies were done using *ex vivo* testing to assess TiO₂, such as Valant & Drobne (2011) which assessed the biological reactivity of TiO₂ by *ex vivo* testing, and Brun et al. (2015), which assessed the titanium dioxide nanoparticle impact and translocation through *ex vivo* gut epithelia.

The *ex vivo* test system employed in the present study provides evidence that gills were moderately affected in the presence of nTiO₂ in water. However, it is also relevant to note that some condition of the bioassays may also affect the normal functions of gills.

5.1. Exposure to nTiO₂ promote osmotic imbalance

The presence of environmentally relevant concentrations of nTiO₂ in the water yielded moderately histopathological lesions, mainly CC autolysis e GC hypertrophy. In fact, the nTiO₂ increased the severity and dissemination of these alterations, as showed by the increment of the I_h relatively to T_0 gill and control treatment, after 2 h of exposure. Representative correlations between the I_h and the respective responsive patterns were found, demonstrating that progressive and regressive alterations were fundamental to increase the histopathological indice (Van Dyk et al. 2009). Goblet cell hypertrophy and epithelial lifting showed high correlations when compared to histopathological indice. Progressive alterations, such as, hyperplasia and hypertrophy are considered protective measures against toxicants (Mallatt et al., 1985), possibly resulting in an unbalanced osmotic regulation, gas exchange and rapid mucous release.

Additionally, the highest concentrations of nTiO₂ (200 µg L⁻¹) induced the highest I_h value at both experiments, being significant at 4 h of exposure. These changes are not considered specific to nTiO₂ exposures, being reported to occur in fish exposed to a wide variety of pollutants from heavy metals to organic compounds, like PAHs (Martins M. et al., 2015, Martins C., 2015, Martins M., et al 2016a). From the few histological studies done so far, Federici, (2007), demonstrated that changes in mucocytes morphology would occur when fish gills were exposed to 0.5 mg nTiO₂ L⁻¹. Federici, (2007), also demonstrated interlamellar hyperplasia. Accordingly, Xia Dong et al., (2011), hyperplasia occurred when fish were exposed to 1, 2, 3 and 4 mg nTiO₂ L⁻¹, which caused fusion of some areas as well as gill injuries were also aggravated when exposed to higher concentration. During our study, it was also demonstrated that hyperplasia was a histopathological alteration, although not too severe or disseminated.

In addition to the histopathological lesions, the number of chloride cells increased in gill exposed to nTiO₂ comparing to control treatments, for 2h. It is well known that these cells are involved in ion transport in order to maintain the osmotic balance. As such, the present results may indicate that the immune system is reacting to the presence of nTiO₂ in the water, producing more CC in gills. In fact, changes in number, size and distribution of CC in teleosts have been attributed to salinity (Karnaky et al., 1976) and also to metals (Giari et al., 2007). However, the results revealed that prolonged exposures, i.e. 4 hours of exposure (T₄) does not imply the increase of CC, in *ex vivo* experiments. This may be attributed to the absence of a systemic biological system when considering *ex vivo*, which may result in a deficit of osmoregulation.

5.2. Alterations in mucocytes reveal response to challenge

Mucous secretion (especially on the gill epithelium) is considered a defensive mechanism against toxicants (Handy et al., 1989). This common response of the mucus layer is excessive mucus production of mucosubstances, like glycoproteins and glycolipids, with the consequent swelling of epithelial cells followed by a change in the number of goblet cells (Bols et al., 2001). In the present study, the number of goblet cells per interlamellar space changed as a response to the exposure to nTiO₂, however the pathway was time-dependent. Whereas the number of GC increased at T₂, prolonged exposure (T₄) decreased the number of this mucocytes. These results suggested a defensive mechanism toward the challenge, while a prolonged exposure to nTiO₂, may overwhelmed the ability of immune system responses to cope with the injury, resulting in a decreasing number of goblet cells. In fact, changes in mucous cell function (number and size of goblet cells) have already been reported in studies when fish were exposed to distinct substances, like metals and organic toxicants (Costa et al., 2009; López-Galindo et al., 2010; Martins et al., 2015; Martins et al., 2016). In addition, excessive mucus production has also been documented in gills of rainbow trout after exposure to carbon based NPs (Smith et al., 2007). This response may be particularly critic in *ex vivo* systems, since they have not the same intercellular interactions as *in vivo* systems, compromising the organism immune-response capability. The consequences of the reduction of mucus cells can enhance the infection potential, reduce lubrication of gill structures with consequent damage and hinder the excretion and regulation of metals and other ions.

5.3. No clear evidence of nTiO₂ uptake from standard histological methods

Regarding metal deposits count, no significant differences were found between treatments or time of exposure. This result point to the problem commonly associated agglomeration of NPs in water. It is well known that NPs tend to agglomerate, especially in salt water, due to the increase of ionic strength which reduces the negativity of electrophoretic mobility of the particles (Batley et al., 2013). In addition, the bioavailability of NPs is influenced by the presence of

organic matter in water, changes in pH and ions (Baker et al., 2014). In fact, the Dynamic light scattering analysis indicated agglomeration of NPs, (data not shown). Probably this fact influenced the toxicity of the exposures since, generally, NPs agglomeration is associated with the increment of NP's size and the decrease of NP's toxicity.

Some authors pointed that NP toxicity may be driven by the surface chemistry (reactivity) of the particles (Shaw and Handy, 2011). Parameters such as pH and light exposure (UV or natural) may modify the physiochemical characteristic of particles and make them more or less biologically active. It is pointed that nTiO₂, when exposed to UV or natural light, due to their photocatalytic activity, can generate radical oxygen species (ROS), possibly inducing oxidative stress in cells (Xiong et al., 2011). In the present study, these parameters were monitored in both experiments and no differences were observed between control and nTiO₂ treatments (data not shown).

Although metal deposits were found in gill macrophages, they were consistently distributed through all treatments. Generally, this response of the immune defence system is efficient in organisms and several NP aggregates will be expected in gill macrophage in *in vivo* experiments. Conversely, *ex vivo* systems may have limited this response. However, other methods, such as Transmission Electron Microscopy (TEM), would added information about this issue.

5.4. *The ex vivo bioassays generated significant confounding factors*

The lesions observed in gills exposed to control treatment (sea water) showed severe epithelial lifting and, also, alterations of the structure of lamellae. The gills, because of their direct and permanent contact with water, are particularly sensitive to adverse environmental conditions (Thophon et al. 2003), including abrupt changes of salinity (Arjona et al., 2007). In addition, they are important organs since they perform vital functions such as gas exchange and ion osmoregulation. Through effective mechanisms of osmoregulation, euryhaline teleosts are able to cope with changes in salinity by the active transport of salts in the gills (Lin et al., 2004). In particular, *S. Senegalensis*, which is an estuarine species, is able to acclimate to different osmotic conditions during short-term exposure (Arjona et al., 2007).

In fact, common alterations recorded in the present study, such as epithelial lifting, epithelial cell proliferation and lamellar fusion, have also been found in studies addressing osmoregulation in fish. Is the example of the *in vivo* work with *Liza aurata* fish after the exposure to acute increase in salinity (Arjona et al., 2007).

This effect is a protective response to salinity stress since reduce gill surface area however, the efficiency of gas exchange will be severely affected. At the same time, changes in salinity also affect mitochondria-rich cells, i.e. chloride cells. The chloride cells of the gill secretory epithelium of euryhaline fish, such as *S. senegalensis*, adapt to the increment of salinity by stimulating chloride secretion, which basically pump salt from the blood to the outside medium and, in a second adaptation, by increasing the number and size of chloride cells (Arjona et al., 2007). In the present study, the gills were exposed to treatments immediately after excision and directly to sea water medium. Probably, these experimental conditions, i.e., *ex vivo* exposure in seawater, triggered acute salinity stress, to which gills responded primarily with the alterations of the epithelial structure, however without significant chloride cell alterations, probably due to the short-time of the experiments (2 and 4 hours). In fact, the second adaptation mechanism takes place after days or weeks to the increment of salinity (Karnaky KJ JR, Kinter LB, Kinter WB, Stirling., 1976).

Ex vivo test reported in this study is well suited to the fast screening of the biological potential of nanoparticles. Nonetheless, important factors should be assessed while preparing an *ex vivo* test, such as the preparations of the solutions in the right environment, salinity and NPs characterization is important to avoid confounding factors.

Nonetheless, some improvements have to be implemented such as (i) the use of nanoparticulate TiO₂, suspended in a physiological solution; (ii) the assess of the cell viability; (iii) the evaluation of the NP agglomeration using TEM (Transmission Electron Microscopy) and evaluation of their shape and size.

6. Conclusions

Overall, findings from this study indicate, that under ecologically relevant concentrations, nTiO₂, can cause moderate effect on gills of *S. Senegalensis* in short-time exposures. However, several parameters, such as pH of the exposure media or the presence of other molecules, may modify the physiochemical characteristic of particles and make them more or less biologically active than original or primary particle characteristics. It is, therefore, required to have a good understanding of the characteristics of particles to better understand their biological potential.

In conclusion, the *ex vivo* tests reported in this study are well suited to the fast screening of the biological potential of nanoparticles, however, several issues have to be taken into account such as the medium for NPs and organ exposure. The data obtained from these experiments may significantly contribute to define further procedures to nanotoxicity studies.

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